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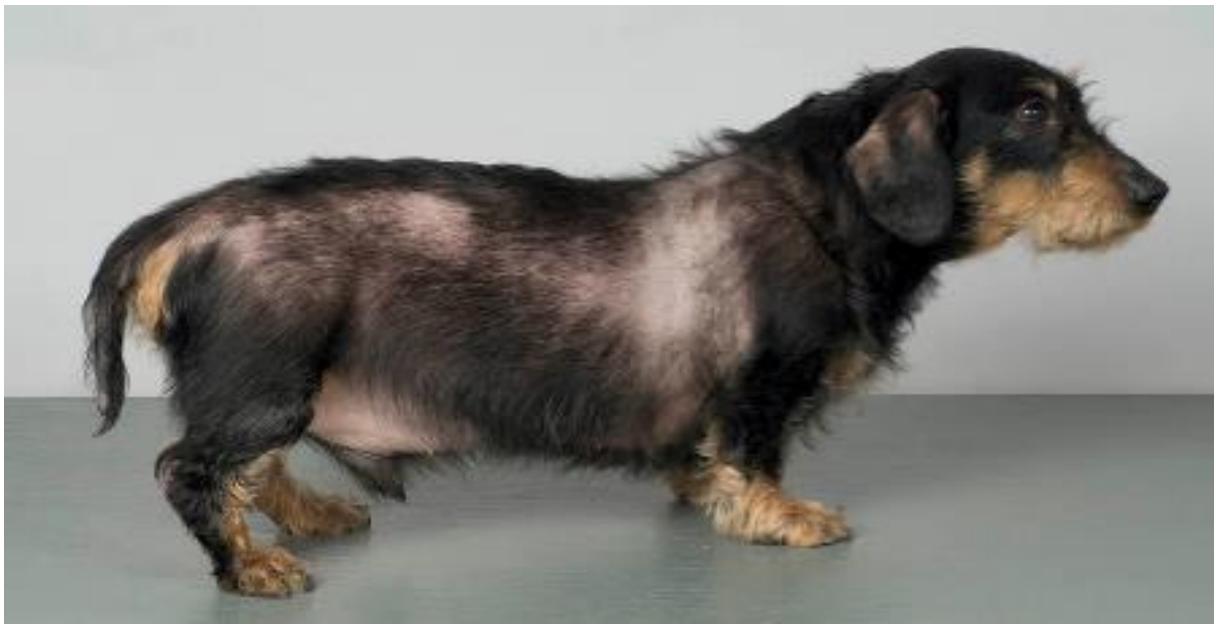
SOX9 and Ki67 as novel prognostic factors in canine corticotroph pituitary tumors



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March 2022
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Abstract

Background

Pituitary-dependent hypercortisolism (PDH) is a common endocrinological disorder in dogs. This disorder is caused by an adrenocorticotropic hormone (ACTH)-secreting adenoma in the pituitary gland. Transsphenoidal hypophysectomy has shown to be an effective treatment for PDH, for which the pituitary height/brain area ratio (P/B ratio) is an important prognostic marker. Additional prognostic biomarkers could help to select dogs that could benefit from additional treatment or monitoring after surgery. The objective of this study was to investigate the prognostic value of stem cell marker sex determining region Y-box (SOX9) and proliferation marker Ki67 in canine corticotroph pituitary adenomas.

Method

Protein expression of SOX9 and Ki67 was analyzed by immunohistochemical stainings in 31 enlarged corticotroph pituitary adenomas of dogs that underwent hypophysectomy between 2015 and 2021. For SOX9 quantification, the H-score was assessed twice by visual scoring with a light microscope. The Ki67 proliferation index (PI) was determined by calculating the percentage of positive nuclei in at least 1000 cells in a hotspot area per tumor slide. Statistical analyses was performed to determine the correlations between the P/B ratio, SOX9 and Ki67. To assess the effect of the prognostic factors on the disease-free survival time, univariate and multivariate survival analyses were performed using the Cox proportional hazards model. The Kaplan-Meier limit-method was used to compare disease-free survival times between groups.

Results

The P/B ratio and Ki67 PI were both significantly associated with survival in univariate survival analyses, and were correlated with each other. The H-score of SOX9 showed a trend towards being significantly associated with poor prognosis after surgery in univariate analysis. Dogs with corticotroph pituitary adenomas that had high SOX9 H-scores or high Ki67 PI's had worse disease-free survival times than those with low scores. The multivariate regression model with forward selection showed that the Ki67 PI and SOX9 H-score were independent prognostic factors for dogs with PDH after hypophysectomy.

Conclusion

This research provided evidence that stem cell marker SOX9 and proliferation marker Ki67 are useful intra-tumoral predictors of recurrence of PDH for dogs after hypophysectomy. These novel prognostic factors are promising markers that can be used to select dogs that would benefit from additional monitoring and/or adjuvant therapy.

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Introduction

The pituitary gland is a fundamental component in the endocrine system. It is located in the pituitary fossa in the basisphenoid bone and is in contact with the base of the brain (1-3). This endocrine gland comprises of the adenohypophysis (consisting of the anterior and intermediate lobe) and the neurohypophysis (consisting of the posterior lobe, Figure 1) (3).

The hypothalamus is connected to the pituitary gland in several ways and mediates the release of various hormones. For example, the hypothalamic-hypophyseal portal system is responsible for the transport of releasing and inhibiting hormones to the anterior lobe (AL) of the pituitary gland. As a result, the AL secretes several peptide hormones by five different cell types, namely thyrotroph, gonadotroph, corticotroph, somatotroph and lactotroph cells. The hormones produced by these cell types affect numerous organs and their physiological processes in a strict way, responsible for metabolism, immunity, body growth, fertility and stress response (3). Dysregulation of the production of these hormones in the hypothalamus-pituitary axis can result in a hormone deficiency or hypersecretion. Pituitary tumors are well-known to cause such dysregulations.

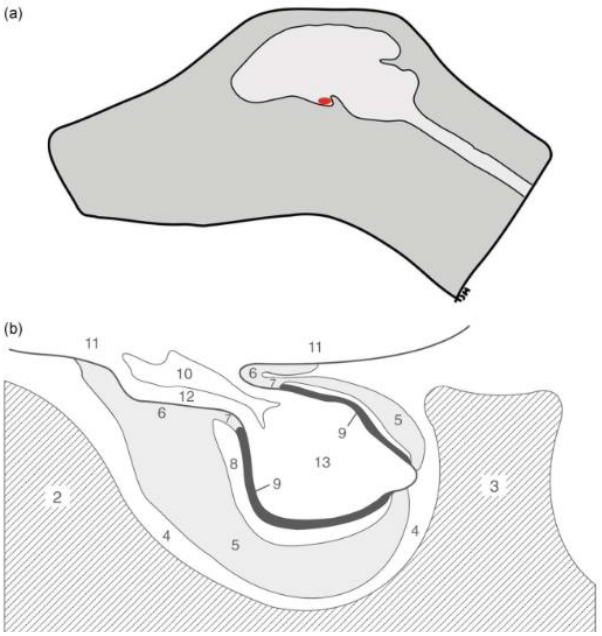

***Figure 1**. Anatomy of the canine pituitary gland. (a) Schematic lateral view of a canine skull. The pituitary gland (red) is positioned in the cranial cavity underneath the brain. (b) Schematic overview of the pituitary gland with the anterior lobe (5), intermediate lobe (9) and posterior lobe (13).*
Taken from: Stem cells in in the canine pituitary gland and in pituitary adenomas, (figure 1, pp. 218). Van Rijn, S.J, Tryfonidou, M. A., Hanson, J. M., Penning, L. C., & Meij, B. P. (2013). Veterinary Quarterly, 33(4), 217-224.

Pituitary tumors are common in dogs and are mostly found in the adenohypophysis (3,4). The vast majority of pituitary tumors in dogs are adrenocorticotropic hormone (ACTH)-secreting adenomas (5,6). Corticotroph cells secrete ACTH and corticotroph adenomas can secrete an excess of ACTH, resulting in an overproduction and release of cortisol in the adrenal glands. These tumors lead to pituitary-dependent hypercortisolism (PDH), which has an incidence of approximately 1-2 cases per 1000 dogs/year (7-9). This makes it one of the most common endocrinological disorders encountered in general veterinary practice. PDH is diagnosed most often in middle-aged to older dogs (7,9). PDH is also known in humans, but has a much lower estimated incidence, namely 1.2-2.4 new cases/million/year (10,11).

PDH in dogs can lead to physical and biochemical changes that are the result of chronic cortisol excess, which results in progressive serious clinical symptoms. It is defined as severe multisystem morbidity with symptoms like polyuria, polydipsia, polyphagia, muscle weakness, bilateral alopecia and abdominal obesity (5,6). Pituitary corticotroph tumors are most often classified as adenomas, but can nonetheless induce space-occupying effects and lead to neurological signs as ataxia, altered behavior and lethargy (6,12). To diagnose PDH laboratory research and diagnostic imaging are necessary, including urinary corticoid: creatinine ratio (UCCR) measurement, dexamethasone suppression tests and diagnostic imaging of the adrenals and pituitary gland (6,13).

There are several treatment options for dogs with PDH, including hypophysectomy, radiotherapy and medical treatment. Factors such as the size of tumor, age of the dog and preference of the owner commonly determine which treatment is indicated (8,13-15). Transsphenoidal hypophysectomy has shown to be an effective treatment, whereby the pituitary adenoma is removed (16,17). However, this is a highly specialized procedure that can only be performed in a limited number of clinics worldwide. Consequently, the majority of the dogs are treated medically, which reduces the clinical signs that are caused by hypercortisolism, but does not inhibit the growth of the tumor. This highlights the relevance for alternative treatment options for dogs with PDH. Currently, there are no registered pituitary-targeting drugs for use in dogs with PDH (8).

Hypophysectomy results in recurrence of PDH in ~23-27% of the canine cases, for which the pituitary height/brain area ratio (P/B ratio) is an important predictive marker. This ratio is determined with CT or MRI. A high P/B ratio is shown to be a negative prognostic indicator (14,15). A pituitary gland is classified as enlarged when the P/B ratio is > 0.31 (18). Another important prognostic indicator is postoperative plasma ACTH concentration. Dogs with an elevated postoperative ACTH concentration were shown to have increased risk of recurrence of PDH (14,19). Van Rijn et al. (2015) provided evidence that a very low plasma ATCH concentration after surgery indicates complete removal of the corticotroph pituitary adenoma (20).

In addition to clinical parameters, intra-tumoral biomarkers can also be associated with prognosis after surgery. The mRNA expression of pituitary tumor transforming gene 1 (*PTTG1)* was previously reported to be associated with disease-free interval after surgery in dogs (21).

Another possible intra-tumoral prognostic factor for canine PDH is the Ki67 proliferating index (PI). Ki67 is a nuclear antigen present in all proliferating phases of the cell cycle (G1, S, G2 and M phases) and therefore associated with the behavior of the adenoma (22-25). In humans, the Ki67 index is a commonly used prognostic factor for corticotroph pituitary adenomas after hypophysectomy.
In canine corticotroph pituitary adenomas, the Ki67 PI has been studied by Van Rijn et al. (2010), who found no association between the Ki67 PI of enlarged and non-enlarged pituitaries, and there was no association between the P/B ratio and Ki67 PI. They concluded that Ki67 was not useful as a proliferative marker for canine corticotroph pituitary adenomas.
However, in this study only seventeen dogs were used, which limits the power of the study. In addition, the published immunohistochemistry pictures show cytoplasmatic staining, while the Ki67 protein is supposed to be present in the nucleus (23,26).

Further research into reliable prognostic factors would help to predict whether recurrence of PDH will occur after hypophysectomy. Knowing which dogs have a high risk of recurrence after surgery, could help to select these dogs for additional monitoring and/or treatment. Studies with mouse models indicated that pituitary stem cell markers could be an important innovative prognostic factor for PDH (27-30).

Stem cells of the pituitary gland

The pituitary gland has a variety of stem cells that are responsible for cell remodeling and differentiation into numerous hormonal cell lineages (31-33). Several cell lineage studies provided evidence that these cells are present in embryonic and adult pituitary glands, which can modify and self-renew in various endocrine cell types (34,35). In addition, stem and progenitor cells are known to be involved in pituitary tumorigenesis, based on study results from mainly murine models (27-30).

A transcription factor that has been reported to be a pituitary stem cell marker is sex-determining region Y-box 9 (SOX9) (Figure 2). SOX9, encoded by the *SOX9* gene, has an important multi-functional role, including development of the pituitary gland, sex determination and the cell differentiation of several hormone-producing cells. In addition to its role in the pituitary gland, SOX9 is also a known stem cell marker in other organs, including the pancreas, kidney and central nervous system (33,36-39). In various malignancies SOX9 is overexpressed and correlated to pathways of tumor cell growth, whereby this transcription factor has shown to be a potential prognostic markers. This include studies with prostate cancer, colon cancer and neurofibromatoma (37,40-42).
Furthermore, overexpression of SOX9 in human growth-hormone (GH) secreting pituitary adenomas was demonstrated in the study of Shirian et al. (2021)(43).

In the anterior lobe of this endocrine gland, SOX2, another stem cell marker, has been studied. SOX2 can be found co-expressed with SOX9, but also represents different stem cells in the adult pituitary gland (33-35). Van Rijn et al. (2015) studied the expression of SOX2 in canine pituitary adenomas, but found no significant association of with prognosis in dogs with PDH. The expression of SOX2 was significantly lower in the tumors (n = 58) than in healthy canine pituitary glands (n = 25) (44). Despite the important function of SOX9 in pituitary development, it's expression has not yet been described in either normal or tumorous pituitary glands of dogs. It would therefore be interesting to determine whether SOX9 protein expression can be used as a prognostic marker in dogs with PDH.

In conclusion, this study aims to identify new markers that can predict the prognosis of dogs with PDH after hypophysectomy. Based on their promising roles as prognostic makers, we studied the protein expression levels of SOX9 and Ki67 in canine pituitary adenomas. We will determine whether there are correlations between the P/B ratio, SOX9 protein expression and the Ki67 PI.

Materials and methods

Case selection

In this study, we used canine normal pituitary glands and corticotroph pituitary adenomas to analyze several markers by performing immunohistochemistry (IHC) and fluorescent IHC.
Normal pituitary glands (n = 6) were collected from dogs that were euthanized for reasons unrelated to the present study, whereby approval from the Ethical Committee of Utrecht University was obtained. The included canine corticotroph pituitary adenomas (n = 31) were collected between 2015 and 2021. All dogs underwent transsphenoidal hypophysectomy at the university clinic in Utrecht as treatment for PDH. All dog owners gave permission to use these tumors for research.

Canine patients with PDH were eligible for inclusion based on the presence of clinical signs and findings in laboratory tests that suggested the presence of hypercortisolism. Hypercortisolism was confirmed by the low-dose dexamethasone suppression test, or elevated urinary corticoid: creatinine ratios (UCCRs) in combination with the high-dose dexamethasone suppression test. When the cortisol concentrations were increased but suppressible by > 50%, PDH was diagnosed. When the cortisol concentrations were increased, but not suppressible by > 50%, differentiation between pituitary- and adrenal-dependent hypercortisolism was based on plasma ACTH concentrations and diagnostic imaging (6,13). Computed tomography (CT) or magnetic resonance imaging (MRI) was performed to visualize the pituitary gland and to determine the pituitary-brain ratio (P/B ratio). Histopathologic reports by the veterinary pathologists confirmed the diagnosis of corticotroph adenomas after surgery in all cases.

Patient and histological parameters

Several parameters of the participating dogs were assessed by reviewing the dogs' medical records, including breed, sex, body weight, age at time of surgery, P/B ratio, and whether and when there was recurrence of hypercortisolism.

Methods

The pituitary adenomas were collected within ten minutes after surgical removal. The tissues were put in one or more containers with formaldehyde. To fixate the tumors, 4% buffered formaldehyde was used for approximately 24 hours. The tissues were subsequently embedded in paraffin. The formalin-fixed paraffin-embedded tissues were cut into 4-µm sections and mounted on SuperFrost Plus microscope slides. For each corticotroph adenoma, one tissue section was stained with hematoxylin and eosin (H&E).

Immunohistochemistry

SOX9 staining

The protocol for SOX9 immunohistochemistry had to be tested and optimized, since the SOX9 antibody (PA5-81966, Invitrogen, ThermoFisher Scientific) had not yet been tested on canine samples. The final IHC protocol was used to stain canine normal pituitary glands (n = 6) and corticotroph pituitary adenomas (n = 31), see Appendix 1. This staining was performed in four batches. As positive control, canine testis tissue slides were used. Negative controls were included by omitting the primary antibody. The resulting staining was approved by a veterinary pathologist (G.C.M.G.). See Figure 2 and Appendix 2 for the results of SOX9 immunochemistry protocol.

For result quantification, the H-score was used. The H-score is a summation of the percentage of cells at each intensity multiplied by the weighted intensity of staining, with a range from 0 to 300 per adenoma (45,46). The positive cells in the corticotroph adenomas were classified on each tissue slide in categories with the intensities of absent (0), weak (1), moderate (2) and high (3) staining. Intensity of staining was determined by comparing with the control testis tissue of that specific batch. The staining intensities were subsequently multiplied by the percentage of cells that had that specific intensity. Only nuclear staining was perceived as positive.

The IHC stainings were qualitatively assessed by the researcher (J.H.) using a light microscope (Olympus BX60 microscope). Beforehand, the researcher was trained by a researcher with experience in pituitary tumors (K.S.). The SOX9 H-score was assessed twice for each tumor at two different occasions. The H&E slides were used to identify pituitary adenoma tissues to make sure that the H-score of SOX9 was assessed only in tumor tissue and not in healthy tissue.

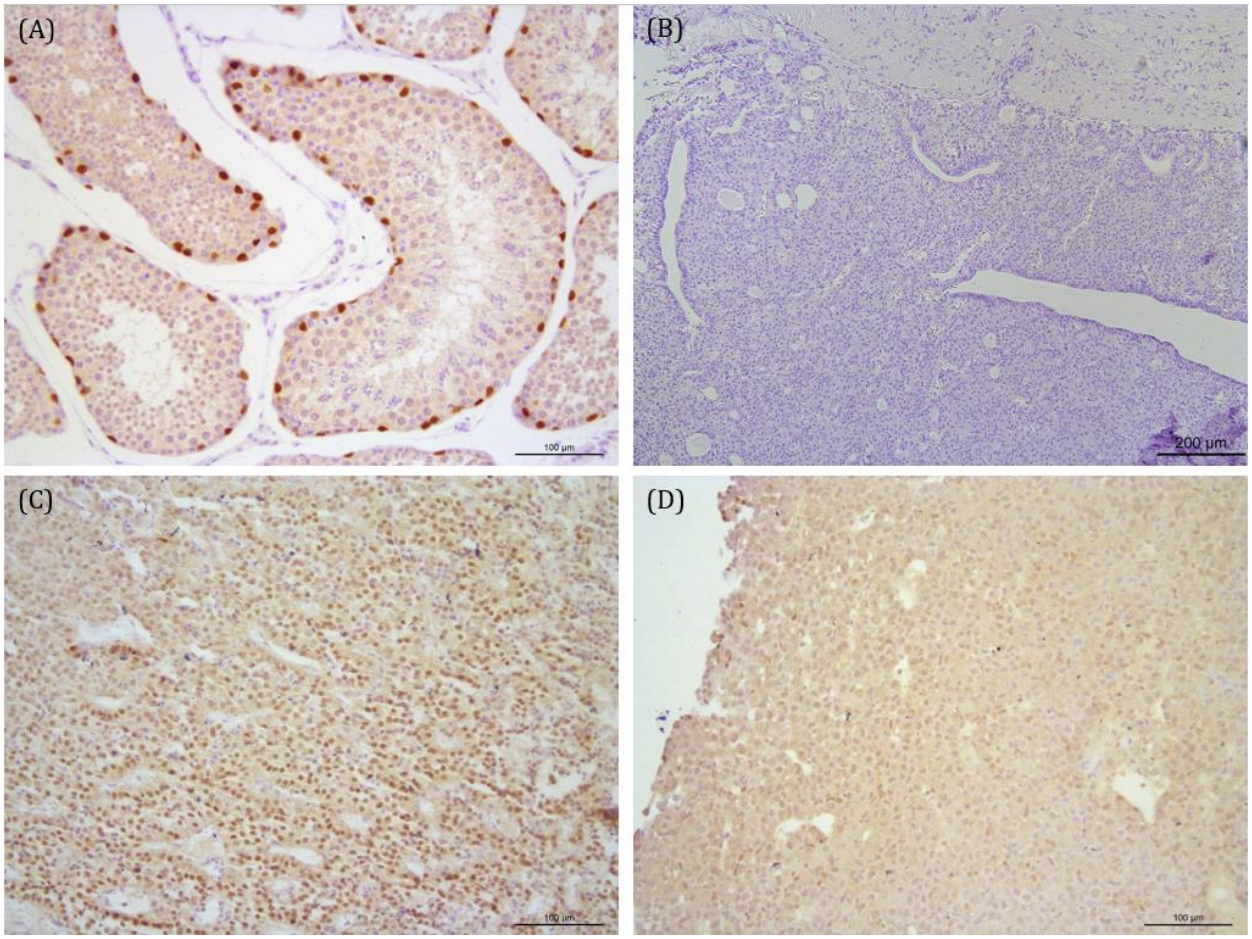

***Figure 2**. Immunohistochemical staining of SOX9. (A). Example of canine testis tissue as positive control with SOX9 positive staining (brown) of the nuclei. Negative nuclei remain blue, bar represents 100 µm. (B). Example of a healthy canine pituitary gland as negative control, staining protocol without the primary SOX9 antibody. Negative nuclei stain blue, bar represents 200 µm. (C). Example of a canine corticotroph pituitary adenoma with an estimated high (92.5) SOX9 H-score. The positive nuclei stain brown and the negative nuclei remain blue, bar represents 100 µm. (D). Example of a canine corticotroph pituitary adenoma with an estimated low (6.5) SOX9 H-score. The positive nuclei stain brown and the negative nuclei remain blue, bar represents 100 µm.*

Ki67 staining

The Ki67 proliferation index (PI) was determined using IHC for all canine corticotroph adenomas. The Ki67 IHC stainings were performed as described previously in the study of Sanders et al. (2019, Appendix 3)(47). This staining was also performed in four batches. As positive control tissue, canine colon tissue slides were used. Canine normal pituitary glands were used as healthy controls. Negative controls were included by omitting the primary antibody. See Figure 3 and Appendix 4 for the results of the Ki67 staining protocol.

To quantify the Ki67 PI, the percentage of positive nuclei was calculated in at least 1000 nuclei in a hotspot area per tumor slide, using Fiji (ImageJ) software to count the positive and negative nuclei. These hotspots were captured at 200x magnification on an Olympus BX60 microscope

with Leica LAS-AF software. For each tissue slide two or more images were taken that contained the highest percentage of Ki67 positive nuclei by estimation. To calculate the Ki67 PI, the number of counted Ki67 positive nuclei was divided by the total number of counted nuclei per corticotroph adenoma.

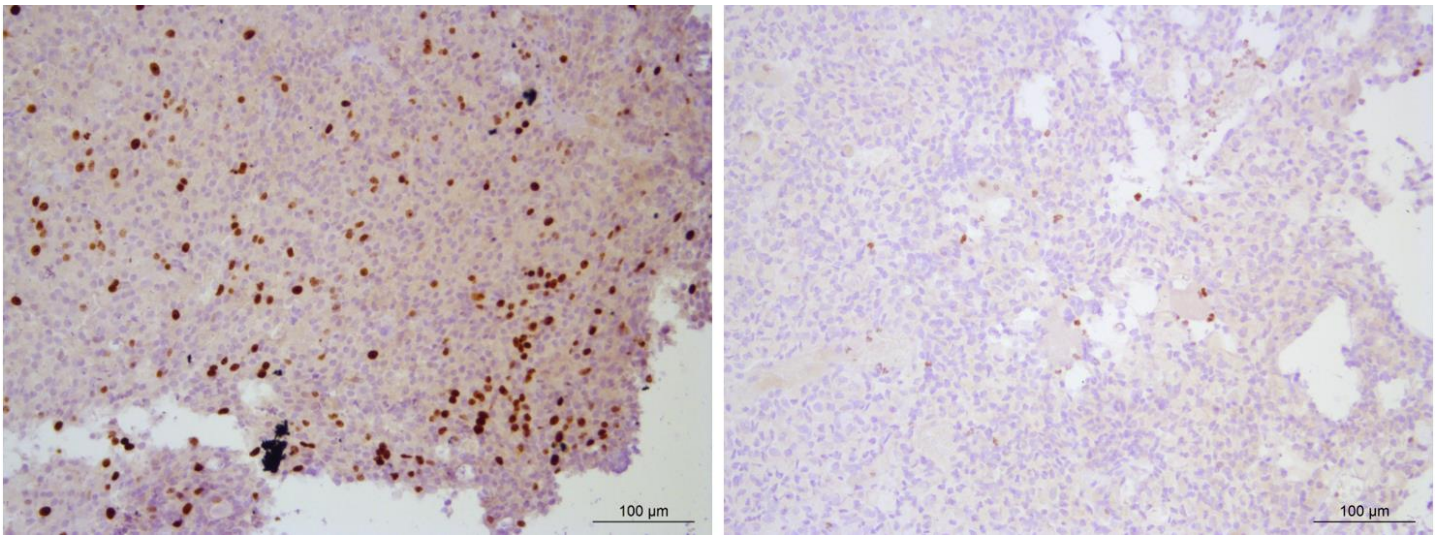

***Figure 3**. Immunohistochemical staining of Ki67. With an example (left) of a canine corticotroph pituitary adenoma with a high (14.6%) Ki67 PI and with an example (right) of a canine corticotroph pituitary adenoma with a low (4.1%) Ki67 PI. Ki67 positive nuclei stain brown and negative nuclei stain blue, bar represents 100 µm.*

Fluorescent immunohistochemistry

In our initial research plan, we wanted to include fluorescent IHC to look at co-expression of SOX9 with Ki67 and other markers. However, the SOX9 staining gave too much fluorescent background to reliably assess protein expression. Thereafter, we decided to only assess SOX9 expression in regular IHC. We have included the protocols used and results of these staining in Appendix 5, 6 and 7.

Statistical analysis

The Shapiro-Wilk and Kolmogorov-Smirnov tests showed that the data were not normally distributed. We therefore used non-parametric test to analyze our data. Correlations between were analyzed using the Spearman's rank correlation coefficient (two-way mixed). Quantification of the intra-observer agreement scores for the H-score was done by using the intra-class correlation coefficient (ICCC), with the following strength of agreement interpretations: <0.00 poor, 0.00-0.20 slight, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 substantial; 0.81-1.00 almost perfect (48).

Dogs were recorded as having recurrence when there was reappearance of clinical signs and UCCRs ratios ≥ 10×10^{-6}, after a minimum of two months after surgery (14). The disease-free survival period was calculated as the interval between hypophysectomy and recurrence of hypercortisolism (event occurred), or as between hypophysectomy and censoring (event did not occur). Patients were censored when they were lost to follow-up, when they died from unrelated causes or when they were still alive without recurrence at the end of the study. The last date of follow-up was used as censoring date. The Cox proportional hazards model was used to analyze the effect of the specific prognostic factors on survival in time in univariate analysis. P-values of

< 0.15 were subsequently included in multivariate stepwise regression using forward selection. The hazard ratio and 95% confidence intervals (CI) were calculated. To calculate optimal cut-off values, receiver operating characteristic (ROC) curves were analyzed. The value with the highest Youden index was selected as optimal cut-off value. Survival analyses were done using the non-parametric Kaplan-Meier method. The log-rank test was used to assess significance in differences in survival times between groups.

Data are reported as median (range). P values of < .05 were considered significant.
IBM® SPSS statistics (version 28.0, Campus license for Windows, 2021) was used for all statistical analyses.

Results

Patient characteristics

In this study, 31 client owned dogs with corticotroph pituitary adenomas were included. Eight of the represented dogs were crossbreed dogs and two were Tibetan terriers. The other dogs were all from breeds that were represented once.
The median follow-up time of the dogs was 387 days (1-1456 days). Several other characteristics of the dogs were collected, shown in Table 1.

Parameter	Distribution (range)
Gender	14 males/17 females
Reproductive status	11 intact/20 neutered
Body weight (kg)	12.9 (3.2-49.9)
P/B ratio	0.62 (0.32-1.32)
Age at time of surgery (years)	8 (4-13)
Recurrence of PDH	6 yes/25 no
Disease-free survival time (days)	387 (4-1456)

Table 1*. Patient characteristics of the included dogs with pituitary-dependent hypercortisolism. Distribution is shown in categories for categorical variables, and in median (with range) for continuous variables.*

Histopathological research was performed on all included canine pituitary tissues. Most of the adenomas were located in the anterior lobe of the pituitary gland. Three tumors were found in the intermediate lobe. The pituitary adenomas presented histological characteristics as monotonous overview of proliferating cells, crude tissue edges and invasive growth.

The estimated median disease-free survival time after hypophysectomy for the included dogs, using the Kaplan-Meier method was 935 days (95% CI: 789-1081 days). Six of the included patients had recurrence of hypercortisolism (Figure 4). The estimated median disease-free survival time for dogs with recurrence was 689 days (95% CI: 0-1430 days). The 25 dogs that had no recorded recurrence were censored in the survival analysis. Therefore, no estimated median disease-free survival time was reached. The median follow-up time was 188 days (range: 1-1456 days).

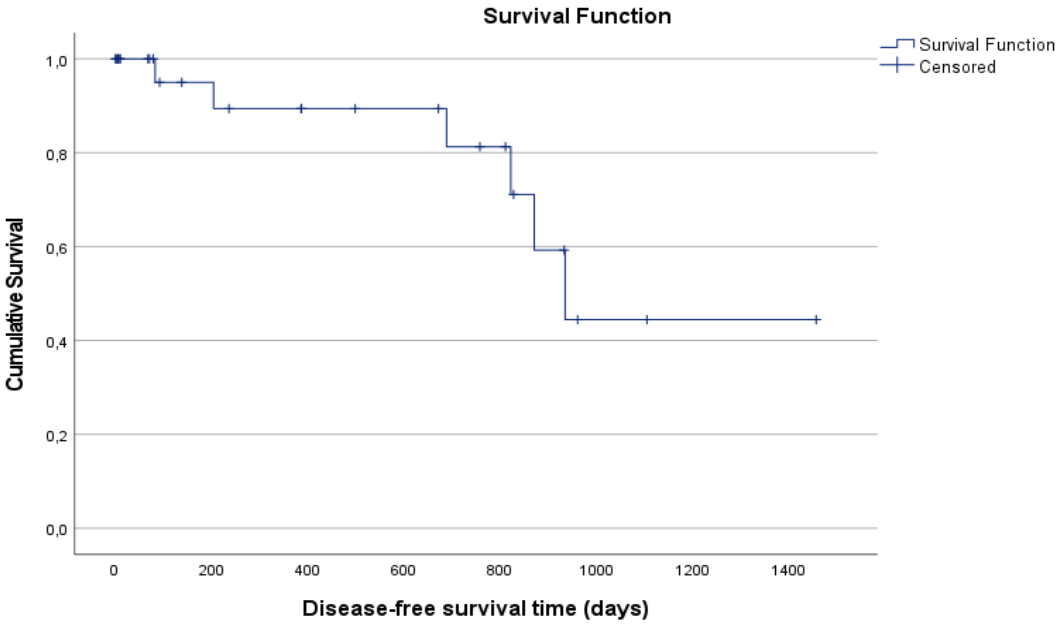

***Figure 2**. Kaplan Meier of the overall disease-free survival time of all dogs included in the study (n = 31). Disease-free survival times were calculated from the time of hypophysectomy to recurrence of PDH or censoring. Censored dogs are stated as tick marks.*

P/B ratio

All dogs had enlarged pituitaries, with a median P/B ratio of 0.62 (range: 0.32-1.32). The P/B ratio was significantly associated with survival (hazard ratio: 17.7, p = 0.049, Table 2). Calculated with the ROC curve and Youden index, the optimal cut-off value for the P/B ratio was 0.67. This gave a significant difference (p = 0.004) between the dogs with a P/B ratio < 0.67 (n = 18, estimated median survival time not reached) and a P/B ratio ≥ 0.67 (n = 11, estimated median survival time of 822 days, 95% CI: 616-1028 days) (Figure 5).

***Table 2.** Univariate Cox regression analyses: effects of prognostic factors on disease-free survival time of dogs with PDH*

Parameter	Median (range)	Hazard ratio (95% CI)	P value
P/B ratio	0.62 (0.32-1.32)	17.7 (1.016-309.116)	0.049
H-score of SOX9	34.5 (4-120)	1.030 (1.000-1.060)	0.051
Ki67 PI (%)	7.9 (1.8-26.5)	1.204 (1.005-1.442)	0.044

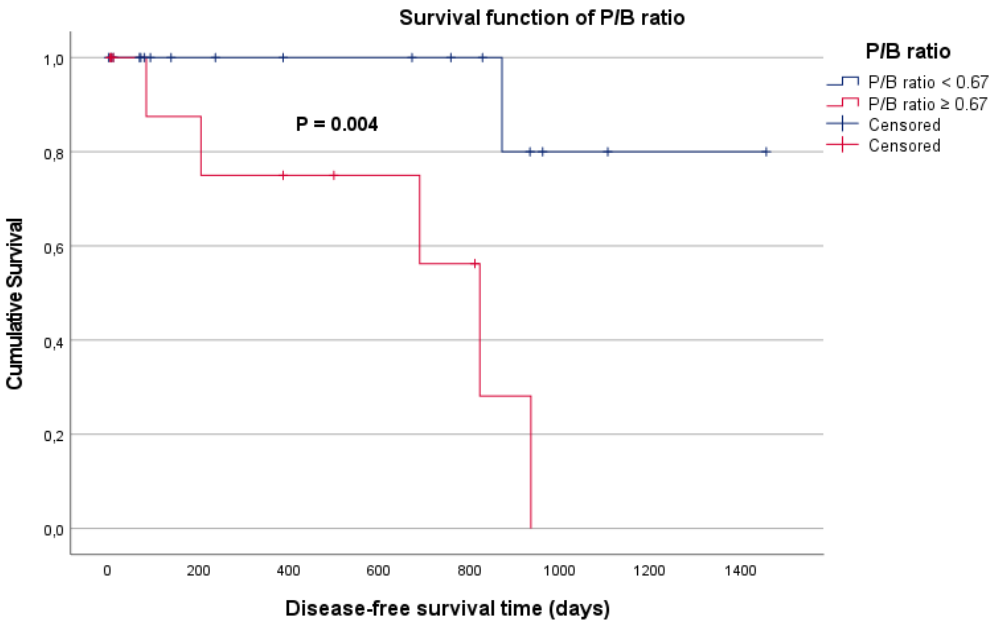

***Figure 3**. Kaplan Meier survival curve of the disease-free survival time in days in dogs with PDH (n = 29). Dogs were classified as having a P/B ratio of < 0.67 (blue line, n = 18), or ≥ 0.67 (red line, n = 11). Disease-free survival times were calculated from the time of hypophysectomy to recurrence of PDH or censoring. Censored dogs are stated as tick marks. The p value demonstrates the significance of the difference between the two groups, using the log-rank test.*

Immunohistochemistry parameters

H-score of SOX9

The intra-observer agreement scores for the SOX9 H-score were quantified by the ICCC and had a score of 0.913 ($p < 0.001$), indicating excellent agreement. The median H-score was 34.5 (range: 4-120).

The SOX9 H-score showed a trend towards being significantly associated with the disease-free survival time (hazard ratio: 1.030, $p = 0.051$, Table 2). For the SOX9 H-score, the optimal cut-off value was 48.25, which resulted in a significant difference ($p = 0.008$) in disease-free survival time between the dogs with a H-score < 48.25 (n = 19, estimated median survival time not reached) and a H-score ≥ 48.25 (n = 10, estimated median survival time of 822 days, 95% CI: 536-1108 days) (Figure 6).

The SOX9 H-score was not significantly correlated with the P/B ratio ($r = 0.154$, $p = 0.407$) or with the Ki67 PI ($r = 0.074$, $p = 0.694$) (Appendix 8).

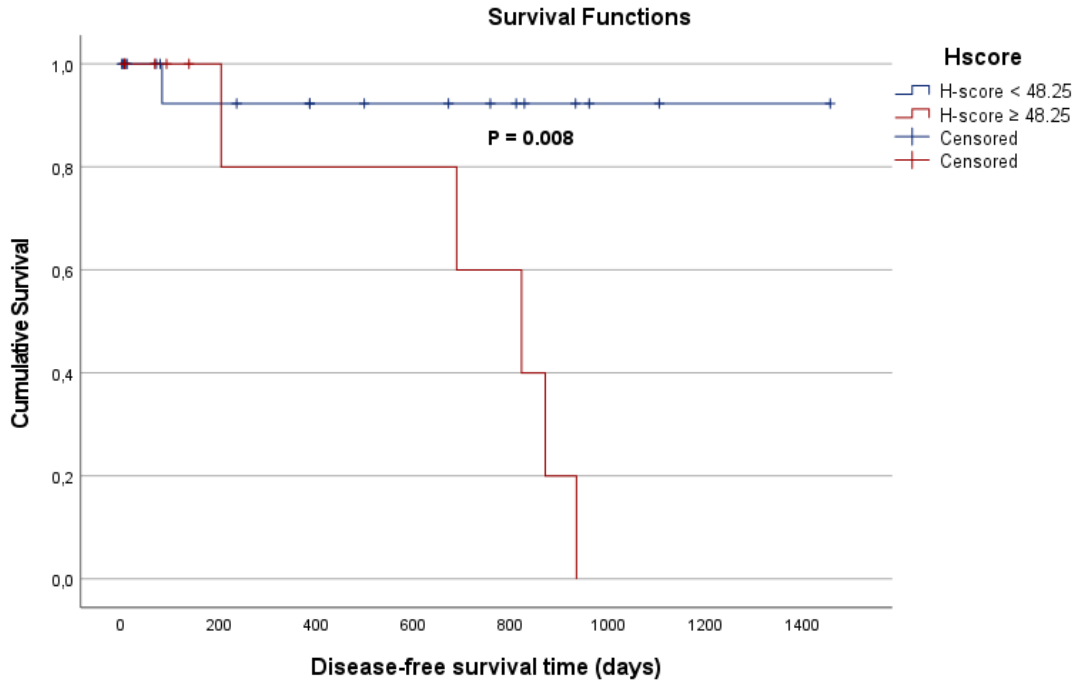

***Figure 4**. Kaplan Meier analysis of the disease-free survival time in days in dogs with PDH (n = 29). Dogs were classified as having a SOX9 H-score of < 48.25 (blue line, n = 19), or ≥ 48.25 (red line, n = 10). Disease-free survival times were calculated from the time of hypophysectomy to recurrence of PDH or censoring. Censored dogs are stated as tick marks. The p value demonstrates the significance of the difference between the two groups, using the log-rank test.*

Ki67 proliferation index

In the included corticotroph adenomas, the median of the counted nuclei was 1101 (range 1014-1542), with a median of 108 Ki67-positive nuclei per adenoma. The median Ki67 PI was 7.9% (1.8-26.5%).

The Ki67 PI was significantly associated with survival (hazard ratio: 1.204, p = 0.044, Table 2). The optimal cut-off value of the Ki67 PI was 11.9, which gave a significant difference (p = 0.014) in disease-free survival times between the dogs with a Ki67 PI < 11.9 (n = 20, estimated median survival time not reached) and a Ki67 PI ≥ 11.9 (n = 9, estimated median survival time 689 days, 95% CI: 221-1157 days) (Figure 7).

The Ki67 PI was significantly correlated with the P/B ratio (r = 0.559, p < 0.001, Appendix 8).

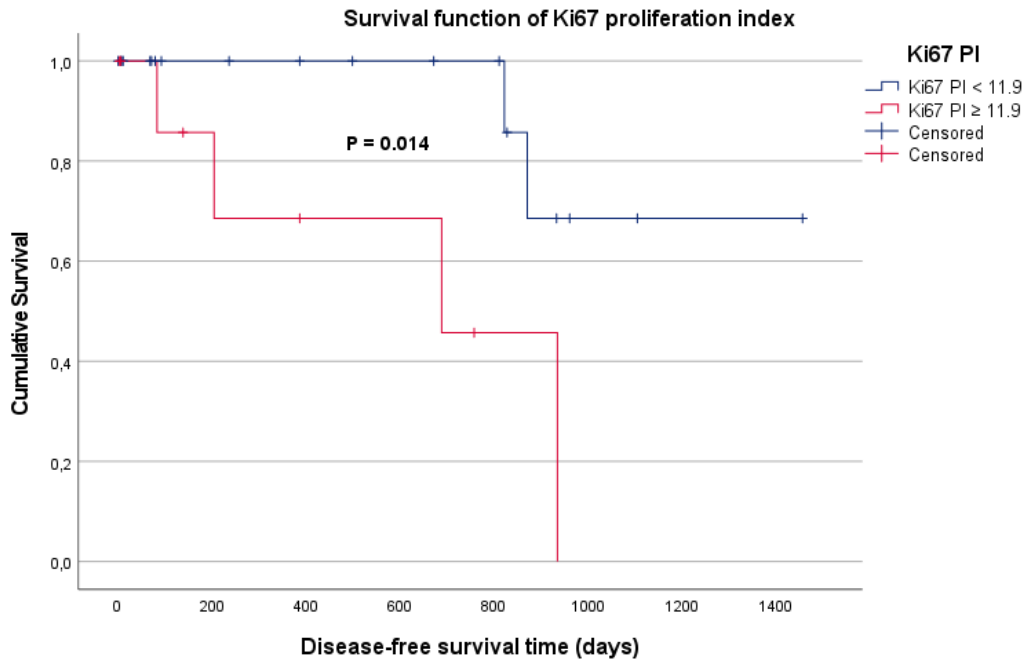

***Figure 5**. Kaplan Meier survival curve of the disease-free survival time in days in dogs with PDH (n = 29). Dogs were classified time as having a Ki67 PI of < 11.9 (blue line, n = 20), or ≥ 11.9 (red line, n = 9). Disease-free survival times were calculated from the of hypophysectomy to recurrence of PDH. Censored dogs are stated as tick marks. The p value demonstrates the significance of the difference between the two groups, using the log-rank test.*

Cox multivariate analysis

The Cox univariate analysis showed that the P/B ratio, SOX9 H-score and Ki67 PI all had p values of < 0.15 (Table 2). Therefore, all factors were included in the multivariate regression model with forward selection to determine if these included factors were independent prognostic factors.

Multivariate logistic regression with forward selection indicated that the Ki67 PI and SOX9 H-score were independent predictors of recurrence (p = 0.014, Table 3). The P/B ratio was not included in the final model.

***Table 3.** Multivariate Cox regression analysis: independent prognostic factors of disease-free survival time of dogs with PDH*

Parameter	Hazard ratio (95% CI)	P value
Ki67 PI (%)	1.199 (1.002-1.434)	0.047
SOX9 H-score	1.035 (0.996-1.076)	0.079

Discussion

The aim of this study was to investigate the prognostic significance of stem cell marker SOX9 and proliferation marker Ki67 in canine corticotroph pituitary tumors. This research showed that SOX9 and Ki67 were intra-tumoral predictors for recurrence of PDH for canine patients, and were demonstrated to have additional value compared to using only the P/B ratio. The Ki67 PI was shown to be a negative prognostic indicator for the disease-free survival time after hypophysectomy. The H-score of SOX9 showed a trend towards being significantly associated with poor prognosis in the univariate Cox proportional hazards model, and showed significant differences in disease-free times between dogs with high (≥ 48.25) or low (< 48.25) intra-tumoral SOX9 expression.

The P/B ratio is an important prognosticator for dogs with PDH and is determined before hypophysectomy (14,15). Here, we demonstrated that dogs with a P/B ratio of ≥ 0.67 had worse disease-free survival times after hypophysectomy than those with a P/B ratio of < 0.67. A recent study of Van Rijn et al. (2016) demonstrated that dogs with a P/B ratio of > 0.31 had an increased risk of recurrence of hypercortisolism, as possible consequence of leaving pituitary tumor cells behind at surgery. However, this research was performed on both enlarged and non-enlarged pituitary glands, in contrast to the current study where we performed research only on enlarged pituitaries. Hence, the optimal cut-off value for P/B ratio may vary when also non-enlarged pituitaries are included (15).
Furthermore, the P/B ratio did not retain its significance in the multivariate analysis. This is presumably caused by the significant correlation between the P/B ratio and the Ki67 PI, whereby the Ki67 PI was more suitable in the best prognostic model for assessing the disease-free survival time after hypophysectomy. However, the P/B ratio is a valuable tool to assess before surgery to have an overview of the size of pituitary gland and shape of the skull, since this varies among individual dogs and dog breeds (2,18). Besides, it is currently the only prognostic factor for dogs with PDH and can therefore play an important role in clinical decision-making.

Identifying additional canine corticotroph adenoma prognostic markers would be useful for dogs with PDH. This information may support to select dogs for additional monitoring and/or adjuvant therapy like radiotherapy.
In the present study, stem cell marker SOX9 was a prognostic indicator for the disease-free survival time after hypophysectomy with an optimal H-score cut-off value of 48.25. SOX9, a member of the SOX-E subfamily, is expressed in stem cells of the pituitary gland and several other tissues. This transcription factor regulates cell renewal and the differentiation into endocrine cells (33,35). SOX9 is overexpressed in various malignancies and correlated to pathways of tumor cell growth, in which this transcription factor was shown to be a prognostic stem cell marker (37,40-42). In this study, SOX9 showed a trend towards being a significant independent prognostic factor for canine patients with corticotroph pituitary adenomas in univariate analysis. When the groups were classified based on having high (≥ 48.25) or low (< 48.25) SOX9 H-scores, the dogs with high scores had significantly shorter disease-free survival times. In addition, multivariate analysis showed that the SOX9 H-score together with the Ki67 PI form a significant prognostic model for dogs with PDH after surgery. This all demonstrates that the H-score for SOX9 has high potency as an independent prognostic factor for canine PDH after hypophysectomy. We therefore expect that the univariate analysis of SOX9 will be significant with a larger sample size and/or longer follow-up time.

The SOX9 staining protocol and quantification of SOX9 with the H-score were performed for the first time on these adenomas. The apparent background staining and only visual scoring of the presence of SOX9 made it difficult to determine the H-score. Therefore, the presence of SOX9 might be under- or overestimated. Additionally, assessment of the SOX 9 H-score was performed twice by one observer. Nonetheless, the intra-observer agreement scores demonstrated excellent agreement, and therefore, intra-observer variability seems to be negligible. In future research, the intra-observer and inter-observer variability of more observers should be studied to assess the feasibility and reliability of SOX9 quantification. However, even with the small sample size and data from one observer of the SOX9 H-score, significant results were assessed for disease-free survival times for canine PDH patients.

In a recent study on SOX9 expression in human GH-secreting pituitary adenomas, SOX9 mRNA expression levels were measured with real-time PCR and the SOX9 protein expression levels were determined with IHC (43). The immunoreactivity evaluation was achieved by analyzing five different hotspots per adenoma, with 200-300 cells per field. The tumor was then assigned to one of following groups: < 5% of neoplastic cells stained positive (no expression), 5-25% of neoplastic cells stained positive (weak expression), 26–50% of neoplastic cells stained positive (moderate expression), and > 50% of neoplastic cells stained positive (strong expression). This study provided evidence that SOX9 was overexpressed in GH-secreting tumors with mRNA analysis and IHC, and the study demonstrated a positive correlation of SOX9 with tumor size and invasion. It was not mentioned how many times this scoring method was performed and/or by different researchers (43). mRNA research on SOX9 on the current database will be performed in the near future. The method for SOX9 quantification of the IHC protocol of the mentioned research of Shirian et al. (2009) could be explored as well (43).

In contrast to the Ki67 expression study of Van Rijn et al. (2010), also performed on canine pituitary corticotroph adenomas, the present study demonstrated that Ki67 is a useful prognostic marker in corticotroph pituitary adenomas. The Ki67 PI was significantly associated with disease-free survival after hypophysectomy. Ki67 PI retained its significance in the univariate and multivariate analysis. These results indicate that Ki67 PI can be used as prognostic factor for recurrence of PDH in dogs. Van Rijn et al. (2010) analyzed the Ki67 PI in seventeen canine corticotroph pituitary adenomas and found no significant correlation between the P/B ratio and Ki67 PI (26). They analyzed Ki67 in enlarged and non-enlarged pituitaries. However, the immunohistochemical staining used in their study showed a cytoplasmatic staining, instead of the nuclear staining that is supposed to be the case for Ki67, since this is a nuclear antigen (23,26). This cytoplasmatic expression of Ki67 might not be associated with cell proliferation and could be a technical issue in the protocol that they used, which may explain the different outcomes.

In the present study, we demonstrated that the P/B ratio and Ki67 PI were correlated. Ki67 is related to the growth and invasive behavior of the tumor (49). Studies on human pituitary adenomas recommend assessing the Ki67 PI as a prognostic predictor of the behavior of the adenoma and surgical outcome (22-25). Ishino et al. (2011) obtained similar results as the current study and demonstrated that the Ki67 expression was related to the P/B ratio in fifteen canine pituitary corticotroph adenomas (non-enlarged and enlarged pituitary glands) (50). In the latter study, the authors quantified the Ki67 expression in at least five microscope fields, by

counting on average 1000 ACTH-positive cells with ImageJ. The percentage of positive stained nuclei was calculated. In the current study, microscope pictures of hot spot areas with the most positive nuclei (by visual estimation) were captured and at least 1000 nuclei were counted. The study of Ishino et al. (2011) and Van Rijn et al. (2010) did not use hotspots and counted on average 1000 nuclei (26,50). For future research it would be interesting to compare the various Ki67 PI quantification methods and to determine which method is the best for predicting the prognosis of dogs with PDH after surgery. Besides, automatic digital image analysis might be possible for future studies to assess the Ki67 PI in corticotroph pituitary adenomas.

The current study showed that a Ki67 PI cut-off value of 11.9% resulted in the classification of two groups that had significantly different disease-free survival times after hypophysectomy. No other canine study was performed in which they determined optimal cut-off values for the Ki67 PI in corticotroph pituitary adenomas. In human corticotroph adenomas there is discussion about assessing a cut-off value for Ki67 as prognostic factor. The most updated issue of the WHO about classification of pituitary adenomas stated that it is recommended to determine the Ki67 PI, but that there is no specific cut-off value of Ki67 PI to predict the aggressiveness of the tumor (51,52). A Ki67 PI of 3% was proposed to differentiate between non-invasive en invasive pituitary adenomas (25). In future studies, it would be interesting to determine whether the Ki67 PI cut-off value of 11.9% is also useful in other patient groups with longer follow-up times. Here it will be essential to use the same methodology as applied in the current study, with more observers to validate this scoring system. Standardization of the immunohistochemical protocol and determination of Ki67 is needed to use the Ki67 PI as a prognostic factor for dogs with PDH after hypophysectomy. Good intra- and inter-observer concordance will support the viability of the current scoring method. Nevertheless, the limitation of this Ki67 PI quantification method is that it may be time-consuming. Future research could focus on digitalization of the process to increase the feasibility and reliability.

The major limitations of the current study were the relatively small sample size and short follow-up time between hypophysectomy and the last available follow-up date. In various included patients hypophysectomy was performed between 2019 and 2021, causing a substantial part of the included dogs to be censored shortly after surgery. For a substantial part of the study population, it was unclear why a patient was lost to follow up.
Another remark is the practical utility of the SOX9 staining protocol and quantification of SOX9 protein expression with the H-score. Future research may investigate if estimation of the SOX9 protein expression by the pathologists is feasible as standard histopathological parameter for canine corticotroph pituitary adenomas, and whether this could be performed on a routine basis.

In conclusion, this study indicated that SOX9 and Ki67 have prognostic value for dogs with PDH after hypophysectomy, and showed added value besides the commonly used P/B ratio. The H-score of SOX9 and the Ki67 PI could therefore help to select high-risk dogs for recurrence of hypercortisolism for additional monitoring and treatment. Future studies should revalidate these outcomes in a larger study population with longer follow-up time and with multiple observers. Having a reliable set of prognostic factors for canine PDH would help to select high-risk dogs that might benefit from additional therapy, for example radiotherapy, which could reduce the risk of recurrence of PDH after hypophysectomy.

Acknowledgements

First of all, I would like to thank my thesis supervisor Karin Sanders for her guidance and inspiring supervision. With her positive feedback I learned a lot about the pituitary gland, laboratory part of veterinary medicine, as well as statistical analysis. This inspired me to do more research in the future.

Moreover, I want to thank Sara Galac for her assistance and insights within the endocrine world. I learned so much from you and your enthusiastic attitude. Thanks to you I could join this research and have this experience outside the clinical world.

Furthermore, I would like to thank all members of the OnGo group. They were very supportive and friendly to work with. I have learned a lot and had a great time as well. And I want to thank Adri Slob especially, for her technical assistance and support with optimizing the staining protocols.

And last but not least, I want to thank my parents and friends for their support and encouragement to develop myself.

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Appendix 1: Immunohistochemistry protocol for SOX9

Sections are counterstained with hematoxylin
Control tissue: canine testis

- Buffer: TBS (pH 7.4) and later TBS 0.1% Tween
- Antigen unmasking solution: citrate (pH 6.0)
- 1st antibody against Sox9 risen in rabbit (PA5-81966, Invitrogen, ThermoFisher Scientific), diluted 1:1000 in 1% BSA in buffer
    - Negative controls are incubated without 1st antibody in the same diluent as the 1st antibody
- 2nd antibody (HRP-labelled goat-anti-rabbit, BrightVision, ImmunoLogic, WellMed, VWRKDPVR55HRP)
- Use DAB substrate kit for peroxidase (ImmunoLogic, WellMed, BrightDAB, VWRKBS04-110)
- Hematoxylin (H-3404, Vector Laboratories, Burlingame, CA, USA), 5 times diluted in buffer
- DPX Merck mounting medium (DPX new, 1.00579.0500, Merck KGaA, Germany)

- 2x5 min xyleen
- 3 min ethanol 96%
- 3 min ethanol 80%
- 2 min ethanol 70%
- 2 min ethanol 60%
- 2x5 min buffer
- Antigen retrieval, 10 min 98°C
- Cool down 20 min on the lab table.
- 2x5 min buffer
- 30 min 3% H2O2 in buffer
- 2x 5 min buffer (1x TBS and then 1x TBS 0.1% Tween)
- Dry slides and take off as much liquid as you can
- Draw circles round section with ImmEdgde pen
- 60 min blocking 10% NGS in 1% BSA in buffer (TBS 0.1% Tween)
- 100 ul, or as much as you need to cover the sections completely, diluted 1st antibody on section in a special made container with a high humidity grade.
- Keep container at 4 °C overnight

Second day

- 3x 5 min buffer (TBS 0.1% Tween)
- Dry slides and take off as much liquid as you can.
- 30 min 2nd antibody at room temperature
- 3x5 min buffer (TBS 0.1% Tween)
- Dry slides and take off as much liquid as you can
- Incubate each slide with 200 µl DAB-solution at RT for 8 min
- 2x2 min demi water.
- Dry slides and take off as much liquid as you can.
- Cover sections for a few seconds with some drops of diluted hemotoxylin
- Drain hematoxylin from slide

- Flush sections during 10 min with tap water
- 2 min ethanol 60%
- 2 min ethanol 70%
- 3 min ethanol 80%
- 3x3 min ethanol 96%
- 2x5 min xyleen
- Dry slides and take off as much liquid as you can.
- Mount a cover slip with mounting medium

Appendix 2: Results of the immunohistochemistry protocol for SOX9

Figure 7*. Microscope pictures a canine pituitary corticotroph adenoma with the SOX9 staining. This is an example of a canine pituitary corticotroph adenoma with an estimated high (92.5) SOX9 H-score. Positive nuclei stain brown and negative nuclei stain blue. (A). Part of the adenoma, bar represents 200 µm. (B). part of the adenoma, bar represents 100 µm. (C). Part of the adenoma, bar represents 50 µm.*

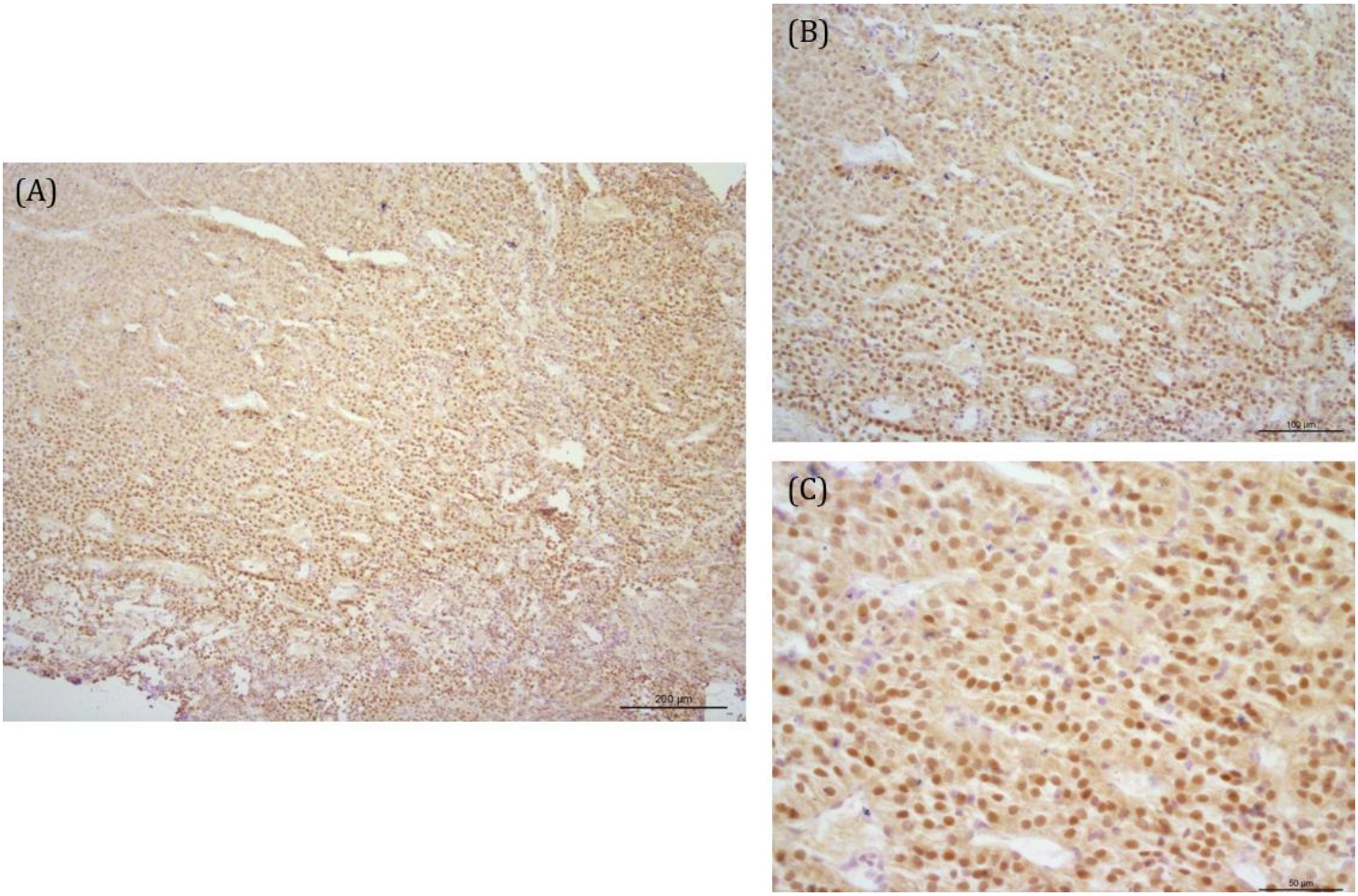

***Figure 8**. Microscope pictures a canine pituitary corticotroph adenoma with the SOX9 staining. This is an example of a canine pituitary corticotroph adenoma with an estimated moderate (56) SOX9 H-score. Positive nuclei stain brown and negative nuclei stain blue. (A). Part of the adenoma, bar represents 200 µm. (B). part of the adenoma, bar represents 100 µm. (C). Part of the adenoma, bar represents 50 µm.*

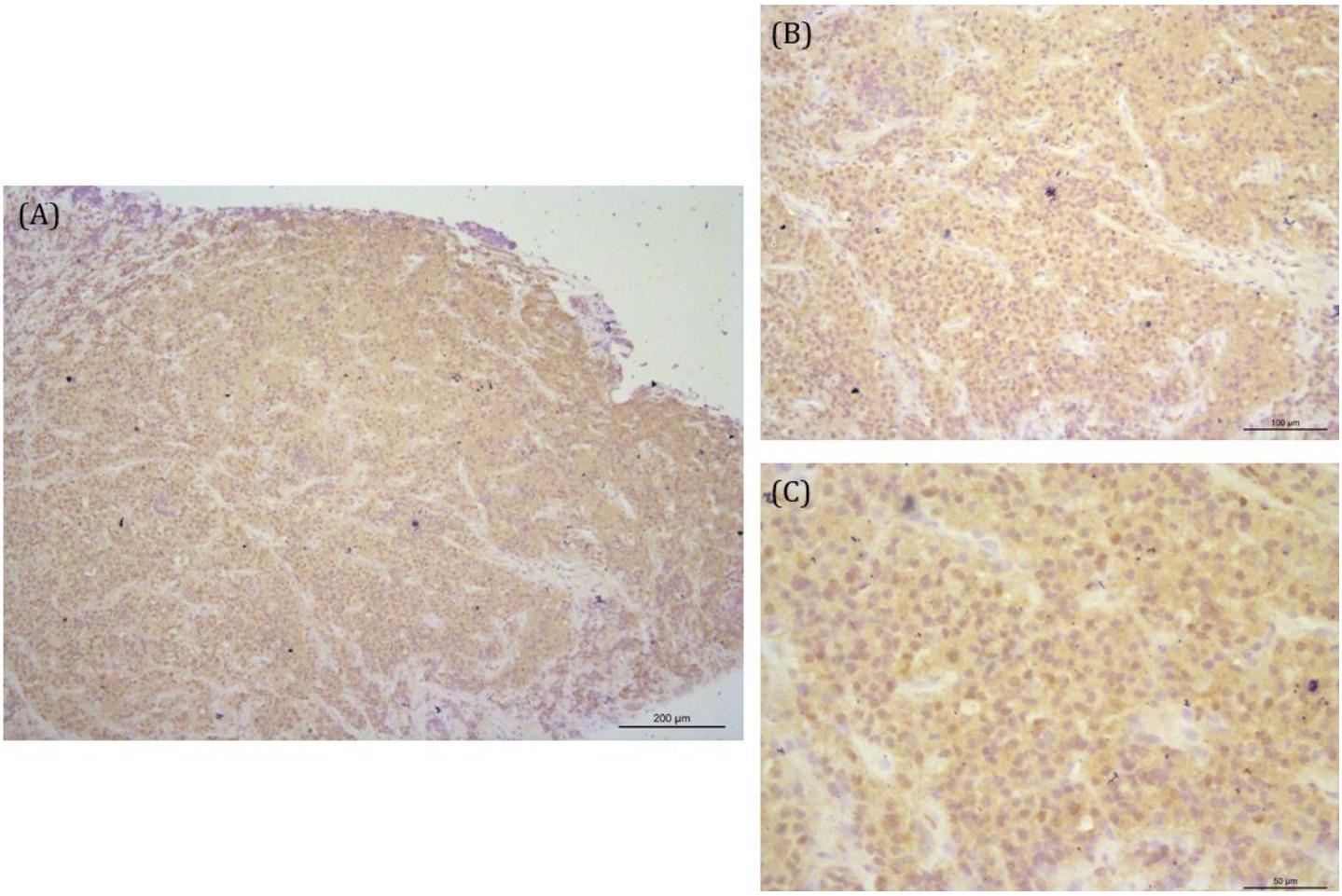

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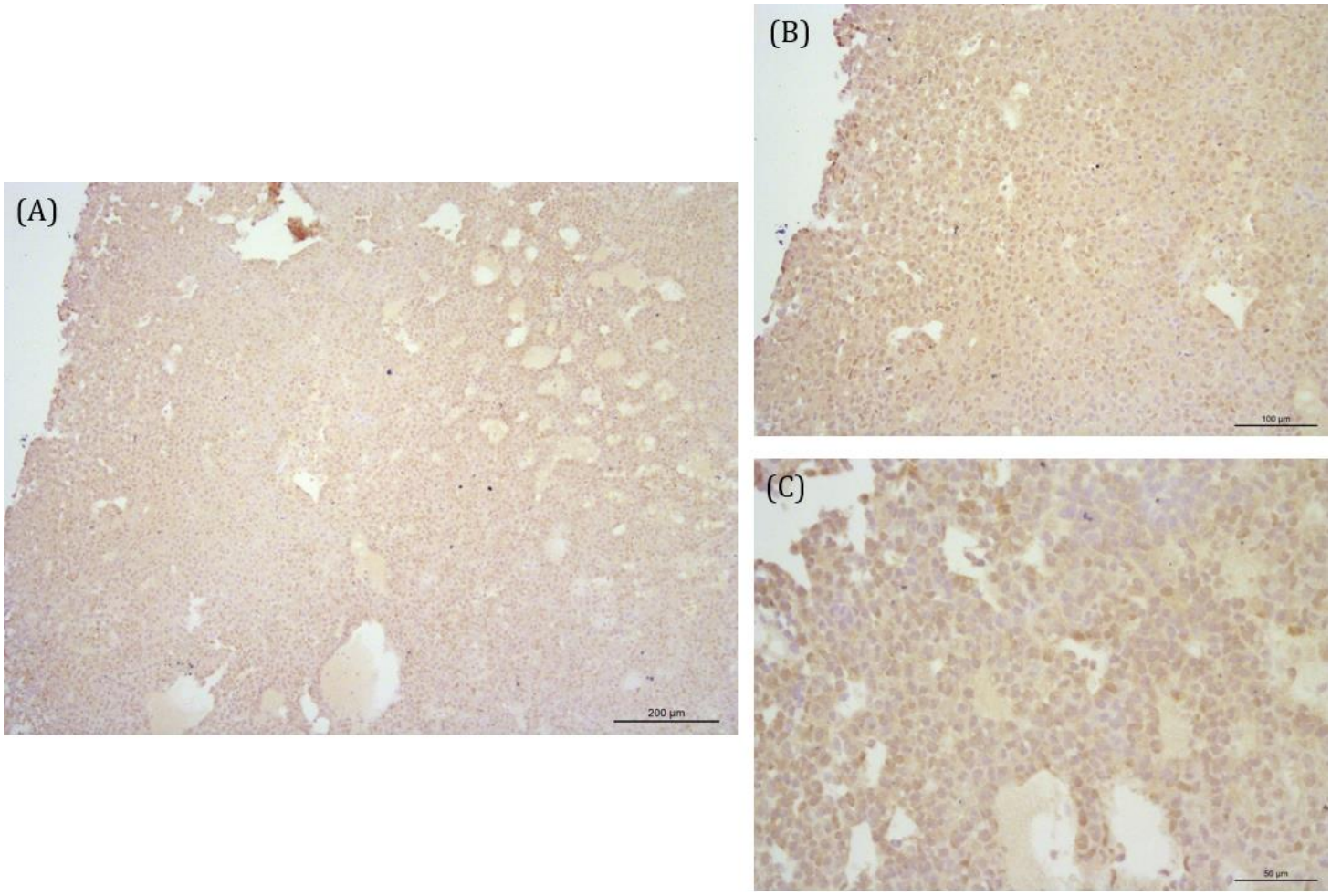
***Figure 9**. Microscope pictures a canine pituitary corticotroph adenoma with the SOX9 staining. This is an example of a canine pituitary corticotroph adenoma with an estimated low (6.5) SOX9 H-score. Positive nuclei stain brown and negative nuclei stain blue. (A). Part of the adenoma, bar represents 200 µm. (B). part of the adenoma, bar represents 100 µm. (C). Part of the adenoma, bar represents 50 µm*.



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Appendix 3: Immunohistochemistry protocol for Ki67

Sections are counterstained with hematoxylin

Control tissue: canine colon

- Buffer: TBS (pH 7.4)
- Antigen unmasking solution: TE (pH 9.0)
- 1st antibody against Ki-67 raised in mouse (monoclonal, MIB-1 clone, M7240, Dako, Agilent, Amstelveen, The Netherlands), diluted 1:75 in 1% BSA in buffer.
    - Negative controls are incubated without 1st antibody in the same diluent as the 1st antibody
- 2nd antibody (HRP-labelled goat-anti-mouse, BrightVision, ImmunoLogic, WellMed, VWRKDPVM110HRP)
- Use DAB substrate kit for peroxidase (ImmunoLogic, WellMed, BrightDAB, VWRKBS04-110)
- Hematoxylin (H-3404, Vector Laboratories, Burlingame, CA, USA), 5 times diluted in buffer
- DPX Merck mounting medium (DPX new, 1.00579.0500, Merck KGaA, Germany)

- Preheat TE solution in microwave (1000 Watt until boiling, a lot will evaporate so make sure to use enough)
- 2x5 min xylene
- 3 min ethanol 96%
- 3 min ethanol 80%
- 2 min ethanol 70%
- 2 min ethanol 60%
- 2x5 min TBS
- Antigen retrieval: 7 minutes 850 Watt, 15 minutes 450 Watt
- Cool down 20 min on the lab table
- 2x5 min TBS
- 20 min 0.35% H_2O_2 in TBS2x 5 min TBS
- Dry slides and take off as much liquid as you can
- Draw circles round section with ImmEdgde pen
- 30 min blocking 10% NGS in 1% BSA in TBS
- 150 µl, or as much as you need to cover the sections completely, diluted 1st antibody on section in a special made container with a high humidity grade.
- Keep container at 4 °C overnight

Second day

- 2x 5 min TBS
- Dry slides and take off as much liquid as you can.
- 30 min 2nd antibody (anti-mouse) at room temperature
- 2x5 min TBS
- Dry slides and take off as much liquid as you can
- Incubate each slide with 150 µl DAB-solution at RT for 10 minutes
- Put the sections in a container with demi water
- Dry slides and take off as much liquid as you can
- Cover sections for a few seconds with some drops of haematoxylin
- Drain hematoxylin from slide
- Flush sections during 10 min with tap water
- 2 min ethanol 60%
- 2 min ethanol 70%
- 3 min ethanol 80%
- 2x3 min ethanol 96%

- 2x5 min xylene
- Dry slides and take off as much liquid as you can.
- Mount a cover slip with mounting medium

Appendix 4: Results of the immunohistochemistry protocol for Ki67

***Figure 10**. Example of a canine corticotroph pituitary adenoma with Ki67 staining. Positive nuclei stain brown and negative nuclei stain blue, bar represents 50 µm.*

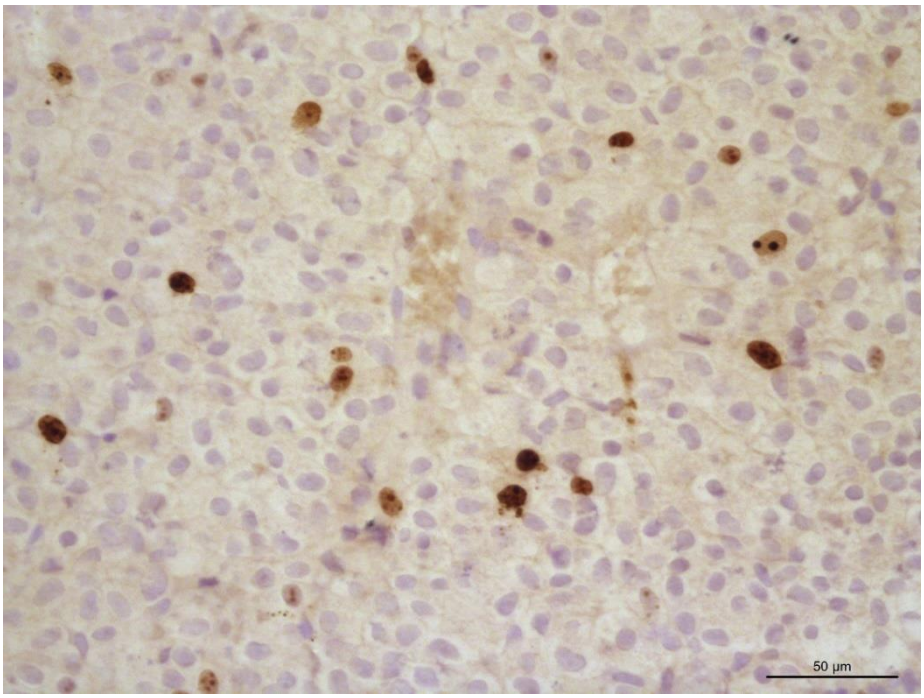
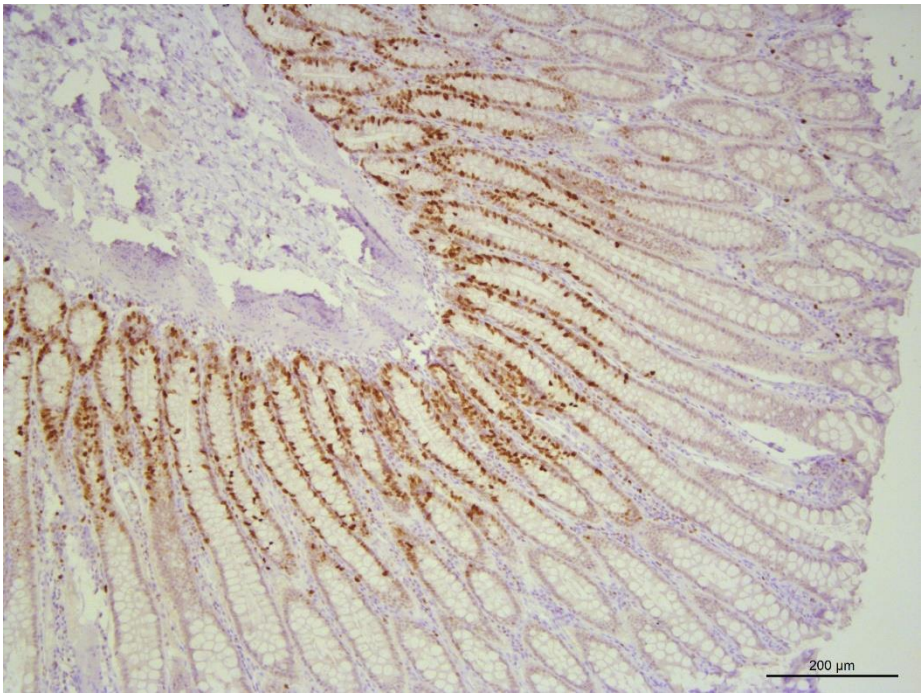


***Figure 11.** Example of canine colon tissue as a positive control with Ki67 positive staining (brown) of the nuclei. Negative nuclei remain blue, bar represents 200 µm.*



Appendix 5: Fluorescent immunohistochemistry protocols co-staining for SOX9 with Ki67 and TBX19

We performed co-staining of SOX9 with Ki67 to determine whether SOX9-positive cells proliferate. This protocol was performed on canine normal pituitary glands (n = 2). We performed co-staining of SOX9 with TBX19 to determine whether SOX9 can be expressed in differentiated corticotroph cells. The following tissue slides were used: canine normal pituitary glands (n = 5), organoids (n = 4) and tumoroids (n = 4).

Organoids, or tumoroids when derived from a tumor, are miniature three-dimensional (3D) structures grown from stem cells, that closely resemble the tissue or tumor they originate from. Organoid and tumoroid cultures are reliable, stable and species-specific *in vitro* systems that allow for strict control and manipulation of study conditions (53,54). Recently, a novel culture protocol was established that successfully induces canine pituitary organoid and tumoroid formation *in vitro* (55).

To test and optimize the co-staining protocols, different buffers (TBS and PBS), antigen retrievals (citrate and Tris-EDTA) and dilutions of the SOX9 antibody were used. There was a significant amount of background staining in both protocols, which made it difficult to identify SOX9 and TBX19/Ki67, and whether they had co-expression or not.
To analyze the co-stainings with SOX9 and Ki67/TBX19 an Olympus BX60 microscope with Leica LAS-AF software was used. The positive nuclei for SOX9 (stained green) and Ki67/TBX19 (stained red) were captured at 400x magnification. Normal nuclei were stained blue.

So, the fluorescent IHC staining protocols were difficult to optimize for SOX9 expression, and especially for the organoids and tumoroids, due to the high background staining. Therefore, we were not able to assess co-expression in the organoids and tumoroids. The fluorescent IHC staining protocol with SOX9 and TBX19 on canine healthy pituitary glands indicated co-expression. With histopathology on organoids and tumoroids, Sanders et al. (2021) provided evidence for resemblance with healthy pituitary glands. The organoids expressed mRNA of pituitary stem cells markers SOX2 and SOX9 (55). Future studies are required to optimize the organoid and tumoroid cultures for use for fluorescent IHC.

Fluorescent IHC protocol description for co-staining of SOX9 with Ki67

Sections are counterstained with DAPI
Control tissue: canine pituitary gland

- Buffer is PBS (pH7.4) OR TBS (pH 7.4)
- To block autofluorescence of erythrocytes: copper sulfate solution (10 mM $CuSO_4$ in 50 mM NH_4Cl, pH 5.0)
- Antigen unmasking solution: TE (pH 9.0)
- 1st antibodies mix against:
    - SOX9 risen in rabbit (PA5-81966, Invitrogen, ThermoFisher Scientific), diluted 1:500 OR 1:1000
    - Ki67 risen in mouse (MIB-1 clone, M7240, Dako, Agilent, Amstelveen, The Netherlands), diluted 1:75
      diluted in 1% BSA in 0.3% Triton X100 in buffer.

- Controls are incubated without 1st antibody in the same diluent as the 1st antibody
- 2nd antibodies mix:
    - Goat anti Rabbit with A488 (Alexa Fluor, Invitrogen by Thermo Fisher Scientific, A11034)
    - Goat anti Mouse with A568 (Alexa Fluor, Invitrogen by Thermo Fisher Scientific, A11004)

  each 1:100 diluted in 1% BSA in 0.3% Triton X100 in buffer
- DAPI (Invitrogen, Thermofisher, Scientific), 1000x diluted with buffer
- FluorSave reagent (Calbiochem, USA, 345789-20Ml).

- 2x5 min xyleen
- 3 min ethanol 96%
- 3 min ethanol 80%
- 2 min ethanol 70%
- 2 min ethanol 60%
- 2x5 min buffer
- 30 min in CuSO4 solution
- Preheat TE solution in microwave (1000 W until boiling, a lot will evaporate so make sure to use enough)
- 1x5 min milliQ
- 3x5 min buffer
- Antigen retrieval: 850 W for 7 minutes, 450 W for 15 minutes
- Cool down 20 min on the lab table.
- 2x5 min buffer
- Dry slides and take off as much liquid as you can
- Draw circles round section with ImmEdgde pen
- 60 min blocking 10% NGS in 1% BSA in 0.3% Triton X100 in buffer
- 100 ul, or as much as you need to cover the sections completely, diluted 1e antibody on section in a special made container with a high humidity grade.
- Keep container at 4 °C overnight

Second day

- 3x 5 min buffer
- Dry slides and take off as much liquid as you can. From now on, the protocol was executed in a dark room.
- 60 min 2nd antibodies mix at room temperature
- 3x5 min filtered buffer
- Dry slides and take off as much liquid as you can
- Incubate each slide with 200 µl DAPI-solution at RT for 10 minutes.
- 2x 5 min filtered buffer
- 1x 5 min MQ
- Dry slides and take off as much liquid as you can.
- Mount a cover slip with mounting medium
- Keep slides in dark, make pictures asap preferably the same day

Fluoroscent IHC protocol description for co-staining SOX9 with TBX19

Sections are counterstained with DAPI
Control tissue: canine pituitary gland

- Buffer: PBS (pH 7.4)
- To block autofluorescence of erythrocytes: copper sulfate solution (10 mM CuSO4 in 50 mM NH4Cl, pH 5.0)
- Antigen retrieval: TE (pH 9.0)
- 1st antibodies mix against
    - SOX9 (PA5-81966, Invitrogen, ThermoFisher Scientific) in rabbit, 1:500
    - TBX-19 (Atlas Antibodies, AMAb91409), 1:500 diluted

  in 1% BSA in 0.3% Triton X100 in buffer.
- 2nd antibodies mix:
    - Goat anti Rabbit with A488 (Alexa Fluor, Invitrogen by Thermo Fisher Scientific, A11034)
    - Goat anti Mouse with A568 (Alexa Fluor, Invitrogen by Thermo Fisher Scientific, A11004),

  all 1:100 diluted in 1% BSA in 0.3% Triton X100 in buffer
- DAPI (Invitrogen, Thermofisher, Scientific) 1000x diluted with buffer
- FluorSave reagent (Calbiochem, USA, 345789-20Ml)

- 2x5 min xyleen
- 3 min ethanol 96%
- 3 min ethanol 80%
- 2 min ethanol 70%
- 2 min ethanol 60%
- 2x5 min buffer
- 30 min in CuSO4 solution
- Dip slides in demiwater
- 3x5 min buffer
- Antigen retrieval, 10 min 98°C
- 20 min cool down on the lab table
- 2x5 min buffer
- Dry slides and take off as much liquid as you can
- Draw circles round section with ImmEdgde pen
- 60 min blocking 10% NGS in 1% BSA in 0.3% Triton X100 in buffer
- 200 ul, or as much as you need to cover the sections completely, diluted 1e antibody on section in a special made container with a high humidity grade.
- Keep container at 4 °C overnight

Second day

- 3x 5 min buffer
- Dry slides and take off as much liquid as you can.
- From this moment, the staining protocol was performed in a dark room.
- 60 min 2nd antibodies mix at room temperature

- 3x5 min buffer
- Dry slides and take off as much liquid as you can
- Incubate each slide with 200 µl DAPI-solution at RT for 10 minutes.
- 2x 5 min filtered buffer
- 1x 5 min filtered MilliQ
- Dry slides and take off as much liquid as you can.
- Mount a cover slip with mounting medium
- Keep slides in dark, make pictures asap preferably the same day

Appendix 6: Results of the fluorescent immunohistochemistry protocol with SOX9 and Ki67

There was a significant amount of background staining in this protocol and the anterior lobe was difficult to distinguish in the canine healthy pituitary glands. There was no difference in the dilution of SOX9 in the intensity of background staining (1:1000 vs. 1:500). It was also not possible to determine whether there was co-expression of SOX9 and Ki67 as a consequence of the background staining. The slides with PBS as buffer were blurry and not useful for assessment.

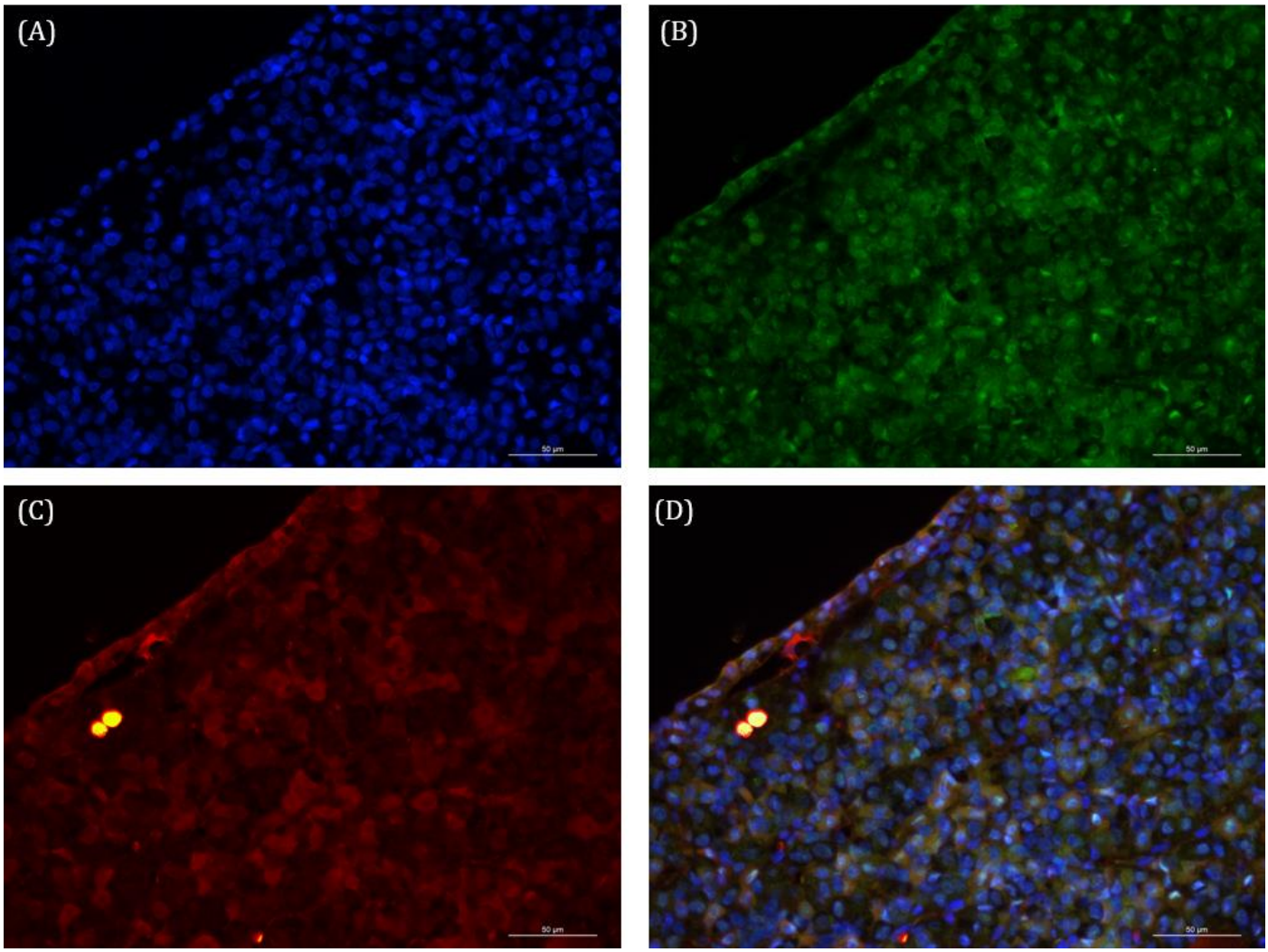


***Figure 12**. Example of canine pituitary gland with the fluorescence IHC protocol of SOX9 combined with Ki67. This protocol was performed with TBS as buffer. (A). Part of pituitary gland with the nuclei stained blue, bar represents 50 µm. (B). Part of the pituitary gland with staining of SOX9, stained green, bar represents 50 µm. (C). Part of the pituitary gland with staining of Ki67, stained red, bar represents 50 µm. (D). Overlay picture of part of the pituitary gland, with staining of the nuclei (blue), SOX9 (green), Ki67 (red), bar represents 50 µm.*

Appendix 7: Results of the fluorescent immunohistochemistry protocol with SOX9 and TBX19

In this protocol there was also a significant amount of background staining. The erythrocytes were strongly visible in the slides. SOX9 was more apparent in the protocol with the 1:500 dilution, in comparison with the 1:1000 dilution.

Antigen retrieval with Tris-EDTA improved the staining protocol. There was less background staining with Tris-EDTA compared to antigen retrieval with citrate.

It is likely that there is co-expression of SOX9 and TBX19 in the canine healthy pituitary glands.

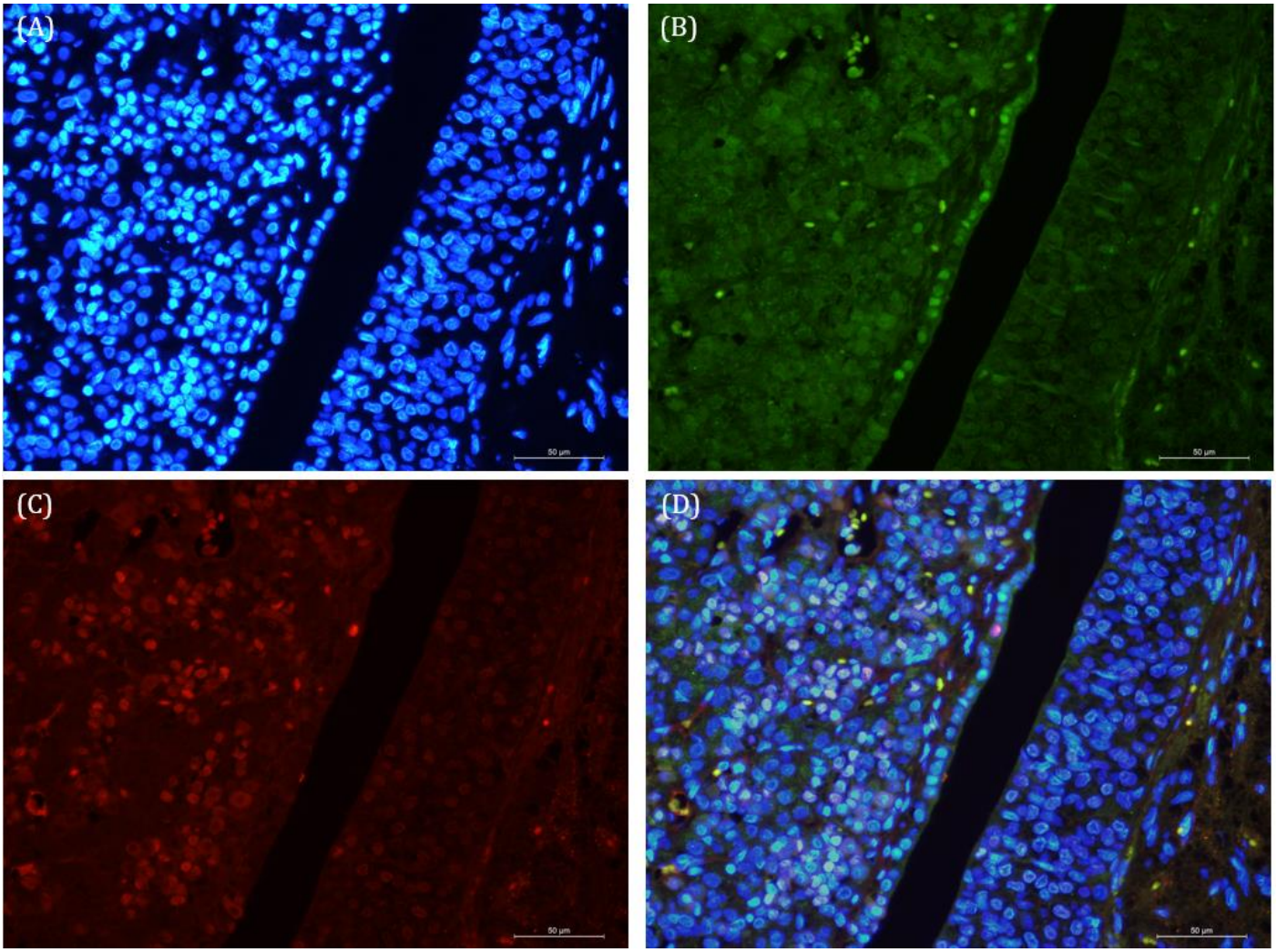


***Figure 13**. Example of canine pituitary gland with the fluorescence IHC protocol of SOX9 combined with TBX19. (A). Part of pituitary gland with the nuclei stained blue, bar represents 50 µm. (B). Part of the pituitary gland with staining of SOX9, stained green, bar represents 50 µm. (C). Part of the pituitary gland with staining of TBX19, stained red, bar represents 50 µm. (D). Overlay picture of part of the pituitary gland, with staining of the nuclei (blue), SOX9 (green), TBX19 (red), bar represents 50 µm.*

Appendix 8 Scatterplots of the correlations of the prognostic factors for PDH

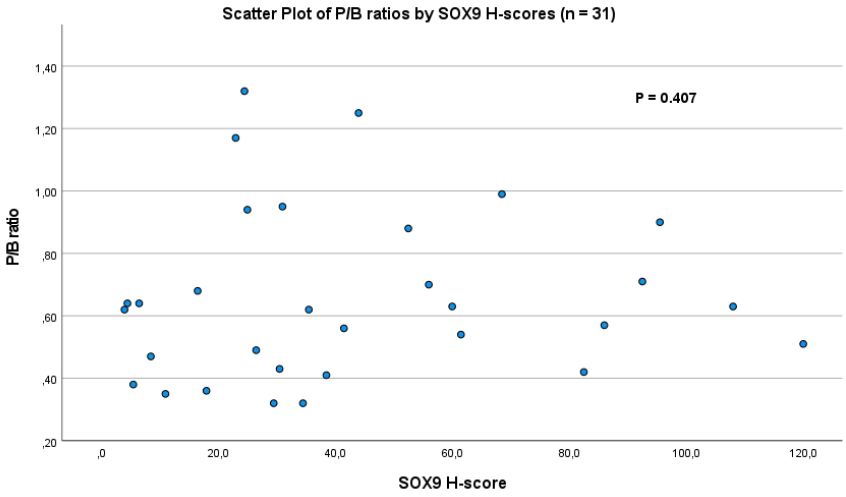


Figure 14. *Scatter plot to show the correlation between the P/B ratio and SOX9 H-score. Each blue dot represents one patient (n = 31). R = 0. 154, p = 0.407*

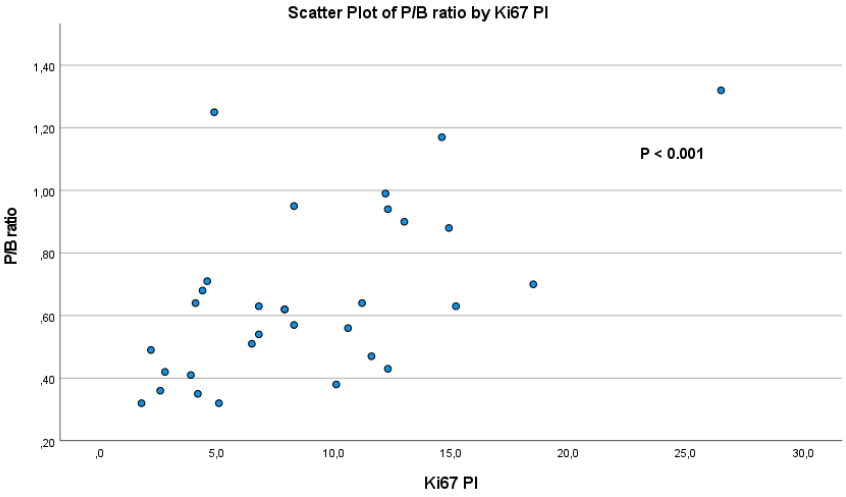


Figure 15*. Scatter plot to show the correlation between the P/B ratio and Ki67. Each blue dot represents one patient (n = 31). R = 0. 5594, p < 0.001*

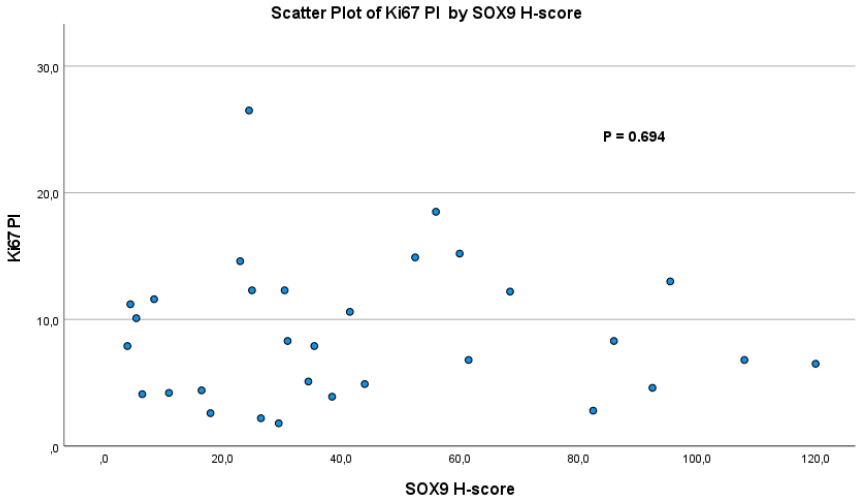


Figure 16*. Scatter plot to show the correlation between Ki67 PI and SOX9 H-score. Each blue dot represents one patient (n = 31). R = 0. 074, p = 0.694*